Environmental stress response in Lactococcus lactis

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RIJKSUNIVERSITEIT GRONINGEN

Environmental stress response in *Lactococcus lactis*: identification of genes and use of expression signals

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te Loenen

Promotor: Prof. Dr. G. Venema

Referent: Dr. J. Kok

VOORWOORD

Hoewel op de titelpagina van dit boek slechts één naam wordt genoemd, is het bewerken van een proefschrift niet iets wat in eenzaamheid gedaan wordt. Graag wil ik daarom op deze pagina's de omstandigheden schetsen waaronder dit boekje gerealiseerd is.

Die omstandigheden worden onder andere bepaald door ogenschijnlijk irrelevante factoren die het werk toch een stuk aangenamer kunnen maken. Zoals de aanwezigheid van een fietspad tussen Groningen en Haren dat steeds verder geasfalteerd werd (en vervolgens met talrijke verkeersregel installaties gebarricadeerd werd), de oprichting van een radiostation dat het presteerde om een maand lang een zingend voorjaarsbos uit te zenden (zeer inspirerend!), en de aanwezigheid van het Noord Willemskanaal.

Andere factoren hebben directer betrekking op dit onderzoek. Zoals de beschikking over een goed toegerust laboratorium met een onuitputtelijke voorraad media en schoon glaswerk. Ik wil dan ook vooral Gerard Venema bedanken voor het geven van de mogelijkheid om mij in alle vrijheid in een goed georganiseerd laboratorium te bekwamen in moleculair biologisch onderzoek. Ten tweede wil ik Arie, Mozes en Peter bedanken, die dag in dag uit daadwerkelijk zorg(d)en voor de schoonmaak van flessen, de bereiding van media en voor een ordelijke voorraad chemicaliën.

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Het project waarvan de resultaten hier beschreven worden was een onderdeel van een groter project met als uiteindelijk doel het ontwikkelen van een induceerbaar lysis systeem. Hierin heb ik de afgelopen jaren samengewerkt met 3 personen. Beste Arjen, Girbe en Harma: Na bijna vijf jaar boven op elkaars lip gezeten te hebben weet ik even niet meer wat daarvan nu wel of niet vermeldenswaardig is. Wel weet ik dat het erg prettig was om met z'n drieën het promotietraject van A tot Z te doorlopen. Verder staan mij nog bij: een aanzienlijk aantal korte(re) en lange(re) discussies, met en zonder eind, zinnig en onzinnig, waaronder het oligosynthese-feuilleton. Onze driemaandelijkse treinreis: degelijke wetenschappelijke discussies in een enigszins oubollige (inmiddels historische) ambiance. Ons gezamenlijk verblijf op zeer uiteenlopende locaties: van hollandse bungalowparken tot aan de andere zijde van dit hemellichaam. Mijn herinnering aan al deze zaken is zeer positief. Ik ben jullie dan ook zeer erkentelijk voor jullie ontspannen collegialiteit en humor. Het grootste gevolg van onze samenwerking ligt (ondanks alle zijwegen) nu in de vriezer: een werkend induceerbaar lysis systeem.

Op labzaal A48 had ik hele andere, maar niet minder gewaardeerde collegae: Kees, als zaaloudste weer teruggekeerd, Albert, toen jij even een primer extensie voor mij deed wist ik nog niet hoe vaak ik dat zelf nog zou doen, Jean, the most lively English teacher I ever met, Marion, Bertus, Marisa en een aantal studenten waaronder natuurlijk Michel Veenstra, Johan de Bondt, Jan Roel Brands en (na een valse start) Jan Burghoorn. Deze laatste vier heren hebben als onderdeel van hun doctoraalstudie aan de stress response gewerkt. Hoewel niet alle resultaten in dit boekje terug te vinden zijn geeft het hoge Jannen gehalte van hoofdstuk 4 aan dat niet alle werk tevergeefs was. Jullie enthousiasme en inzet was hoe dan ook onvergetelijk.

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Zo, nu ben ik eindelijk van school.
Voor jou rest de schone taak dit eindprodukt even door te lezen.
Begin maar bij hoofdstuk 7.....

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Chapter 1

Introduction

INTRODUCTION

Lactococcus lactis has been associated with food production and preservation since ancient times. Food production by fermentation with this organism has always been a significant activity and the scale of fermentations has gradually increased. Nowadays, defined starter cultures of *L. lactis* are of great economic importance in the bulk production of cheese. Apart from milk, which is a nitrogen and carbon rich substrate, *L. lactis* is commonly found in various plant materials, making grass a likely source of inoculation of raw milk (74). Both on plant material and in industrial processes *L. lactis* encounters a wide range of different conditions such as extremes in temperature, pH, or osmotic pressure. The organism is subject to relatively high temperatures in soil or during “cooking” (40?C) in cheddar cheese production. Low temperatures occur during storage of frozen starter cultures and during cheese ripening (8-16?C). Osmotic pressure can vary from very low in rain water to high in pressed cheeses containing 0.56 M NaCl.

Many of these stress conditions will often coincide. This is, for example, the case with carbon starvation and acid stress, as sugar fermentation results in high levels of lactic acid. Dried cells in lyophilized or spray dried starter cultures suffer from both osmotic and oxidative stress. Cells are exposed to both high temperatures and UV radiation in sunlight. Optimal growth conditions are very rare and can, therefore, also be seen as a stress condition requiring specific metabolic adaptations (33).

L. lactis has been studied extensively and is the genetically best characterized species of the lactic acid bacteria. It has obtained the Generally Regarded As Safe (GRAS) status because of its long history of safe use in human consumption. That makes *L. lactis* an interesting tool for the production of new fermented food products or for *in situ* modification of foods. However, the currently known strains can only be used for a limited number of specific applications, as they were primarily selected on the basis of standardized high quality and reliable fermentation of milk. Strains with new traits can be obtained either by selection from natural sources or by genetic modification. The latter approach is more targeted and has opened the possibility for the introduction of genes from non-lactococcal sources, thereby opening the way for specific new applications. Sophisticated techniques for the genetic modification of *L. lactis* have been developed in the last 15 years (11). Cloning vectors based on lactococcal plasmids (both of the rolling circle and ?-replication type) have been designed. In addition, systems have been developed to prevent loss of instable plasmid-encoded traits by integration of genes in the chromosome (6, 51). Promoters, terminators as well as

translation signals, to be used for the expression of any protein of interest, have been isolated. Export signals for efficient secretion of proteins have also been characterized (67,75). All these elements enable recombinant gene expression at the desired (high or low) level and targeting of the gene products to the desired location (the cytoplasm or the medium) (11). In addition, food grade selection markers became available quite recently. These are either based on complementation by a plasmid-located wild-type copy of a mutated essential gene on the chromosome (13, 53, 68) or on the introduction of genes needed for the fermentation of sucrose, which is normally not fermented by *L. lactis*, and selective growth on sucrose containing medium (43). These markers allow gene expression in *L. lactis* by means of systems fully based on lactococcal DNA.

In the (near) future strains containing these systems may be used in the food industry to improve flavour and to reduce production costs, for example, by acceleration of cheese ripening. Other industrial demands to be met are, in general, stable food quality and the availability of strains with stable traits. Techniques for the specific mutation of genes on the chromosome by double cross-over recombination have become available (52) and can be applied in the engineering of metabolic pathways (12). The availability of appropriate genetic systems enables to study the possibilities of using *L. lactis* in a completely new field: as an antigen carrier (84). This would allow vaccination by oral administration of an antigen presenting, but otherwise safe, microorganism.

The gene expression systems developed thus far direct, with a few exceptions, constitutive expression. Inducible gene expression systems are desirable for a number of reasons. Such expression systems are, of course, indispensable for the expression of lethal gene products but they are also preferred in many cases in order to obtain high yields of non-lethal proteins. The induction of certain activities during an (industrial) process requires expression signals that allow tight control. The inducing signals should be food compatible and should, thus, be either a safe food-additive or a change in an environmental condition that occurs naturally or during the fermentation process, or can be easily incorporated in the process. In other words, regulatory systems that respond to changes in the environment that normally control stress adaptation mechanisms hold promise for use in food grade inducible gene expression systems. *L. lactis* is a primary candidate for use as a live recombinant (micro)organism for human consumption. However, present legal barriers and consumer acceptance restrict the use of such strains (81).

In this chapter an overview will be given of the known responses of *L. lactis* to various stress conditions. In addition, the inducible gene expression systems currently available for *L. lactis* will be discussed followed by a brief introduction to the experimental chapters.

2. STRESS

The study of stress responses in *L. lactis* has gained interest in recent years not only because of its industrial relevance but also for pure scientific reasons. Scientific interest is fueled by the fact that *L. lactis* is a mesophilic Gram-positive microorganism with a relatively small genome (2.5 Mbp, 50) that is unable to differentiate in response to stress conditions by sporulation or competence development. Gene regulation in response to stress conditions is very complex and can be influenced at all steps of gene expression such as transcription and translation efficiency, or the stability of mRNA's. In addition, regulation can take place by post-translational modifications. For example, the activity of a gene product may be influenced by its phosphorylation state. Regulation can also take place by differential degradation of a protein. Key examples of stress proteins which are controlled at these various levels are ?^{S} (49) and *cspA* (4, 24) in *Escherichia coli*.

The influence of stress conditions on *L. lactis* has been studied globally by analysing their effects on growth and total protein synthesis (see Table 1) and by genetic analysis of known stress-related genes (summarized in Table 2). In the next paragraphs both global effects and genetic studies will be discussed for a number of stress conditions. Comparison of the different studies in this field is hampered by differences in the analysis techniques and by differences in challenge conditions and incubation times that have been used to stress cells.

TABLE 1. Global response to environmental stress in *L. lactis.*

Stress	conditions	number of proteins:		reference
		induced	repressed	
heat shock	42?C, 5-25 min	12-16	most	85
low temperature	8?C, 1-10 h	12	17 (at 16?C)	64
low pH	pH 5.5, 30 min	33	majority	29
starvation	3 h galactose and arginine exhaustion in CDM[a]	14	>45	48
UV-light	254 nm, 100 J/m^2	14	>30	28

[a] CDM, chemically defined medium

2.1 HEAT SHOCK

Growth arrest was observed when an *L. lactis* culture was shifted from its optimal growth temperature of 30? C to 42? C (85). Growth resumed after a shift-down to 30? with the growth rate reaching the preshock level after 1 hour at 30? C. Cells were hardly capable to recover from exposure to 50? C for 30 min. In this case growth only resumed after 48 hr. The number of viable cells was slightly reduced after a 30-min heat shock at 42? but was more than 1000-fold reduced by incubation at 50?C. However, pretreatment at 42? for 10 min resulted in survival of more than 10% upon heat shock at 50?C (85). Increased expression of 12 to 16 proteins was observed upon exposure of *L. lactis* to 42?C (3, 85). This phenomenon has been observed in organisms as diverse as plants, animals and bacteria. Detailed immunological and genetic analyses have confirmed the presence of the conserved heat shock proteins (HSPs) DnaK, DnaJ, GroEL, GroES and GrpE in *L. lactis*. These HSPs are protein chaperones that function in folding and maturation of new or denatured proteins (21). The organization of the genes encoding these proteins in *L. lactis* is different from other bacteria. Whereas in most cases *dnaK* and *dnaJ* form one operon, lactococcal *dnaJ* is transcribed independently from *dnaK* (2, 79). *dnak* is part of a gene cluster that is also found in other Gram-positive bacteria and consists of ORF1-*grpE-dnaK* (17), followed by a putative transcription terminator and ORF4 that is not induced at high temperature (2).

In a recent study, Arnau *et al*. (2) integrated data from several groups by analysing the expression of 7 genes at the RNA level in response to a standardised heat shock in a single strain. They showed that after 15 min at 42?C *dnaK*-specific mRNA levels in *L. lactis* MG1363 had increased 100-fold and that it represented the single *dnaK* transcript. The levels of ORF1 and *grpE*-specific mRNAs reached a 5-fold higher level after 10 min heat shock and their sizes were similar, indicating that these genes are transcribed together. On the basis of the nucleotide sequence, a promoter could be identified upstream of ORF1 (see below) but not upstream of *dnaK* (17). The discrepancy between this observation and the observed lengths of the mRNAs may be explained by efficient processing of a single messenger covering ORF1-*grpE-dnaK* (2) and differential stability of the processing products. Indeed, larger mRNA's could be detected both with *grpE* and *dnaK* probes. A *groEL* probe hybridized with a 2.2-kb transcript that showed a 10-fold induction after 15 min. This transcript may also include *groES*. The mRNA levels of *dnaJ*, *dnaK*, ORF1 and *grpE* had decreased significantly 20 min after the onset of the heat shock. Only the level of *groEL* transcript was lower at this time

than its normal level at 30? C. The expression of heat shock genes upon induction in rich medium was generally lower and faster than in defined medium (2).

Upstream of ORF1, *dnaJ* and *groESL* vegetative promoters are present which are accompanied by repetitive sequence elements. This repetitive element (CIRCE) is a highly conserved inverted repeat, consisting of 9 bp separated by a 9-bp spacer and was found upstream of heat shock genes in 27 different bacterial species (31).

TABLE 2. Genes induced by environmental stress in *L. lactis*

Gene	Function of protein	Stress condition		Induction factor	Analysis method	Reference
groESL		heat shock	43?C 15 min. in GSA[a]	10	RNA	2
groES	chaperone	heat shock	42?C 30 min. in GM17[a]	12	protein	30
		low pH	pH 5.5, 30 min. lactic acid	3.8	protein	30
		UV-light	254 nm, 100 J/m^2	2.3	protein	30
groEL	chaperone	heat shock	37?C 60 min. in GM17	3	protein	16
		heat shock	42?C 30 min. in GM17	4.9	protein	30
		low pH	pH 5.5, 30 min. lactic acid	2.4	protein	30
dnaJ	chaperone	heat shock	42?C 10 min. in WP[a]	3 to 4	RNA	79
		heat shock	43?C 15 min. in GSA	10	RNA	2
ORF1	negative regulator of heat shock genes	heat shock	43?C 15 min. in GSA	5	RNA	2
grpE	chaperone	heat shock	43?C 10 min. in GSA	5	RNA	2
		heat shock	37?C 60 min. in GM17	3.7	protein	16
dnaK	chaperone	heat shock	43?C 15 min. in GSA	100	RNA	2
		heat shock	37?C 60 min. in GM17	3.4	protein	16
		low pH	pH 5.5, 30 min. lactic acid	2.1	protein	29
ftsH/hflB	regulator of heat shock response	heat shock	43?C 10 min. in GSA	+[b]	RNA	2
		heat shock	37?C 60 min. in GM17	5.2	protein	16
		low pH	pH 5.1, 15 min	5 to 6	RNA	78
recA	SOS regulator ?	DNA damage	0.01% methylmethanesulfonate,	3 to 5	RNA	14
fpg	DNA repair ?		or 1 ?g/ml mitomycin C			
		oxidation	aerated culture	+[c]	RNA	14
sodA	O_2^- scavenging	oxidation	aerated culture	2	reporter[d]	chapter 2
cspA		cold shock	10?C, 4 hr in GM17	40	RNA	86
cspB		cold shock	10?C, 2 hr in GM17	8	RNA	86
cspC		cold shock	10?C, 4 hr in GM17	30	RNA	86
P170		low pH	growth at pH 5.2 vs. pH 7.0	50	reporter	35
		low temperature	growth at 15?C vs. 30?C	7.5	reporter	35
gadCB	acid stress resistance	chloride	growth with 0.5 M NaCl vs. no NaCl	1000	reporter	chapter 4
		low pH	growth + 0.3 M NaCl, in non-buffered vs. buffered M17, pH 4.2 vs. pH 5.5	10	reporter	chapter 4

[a] GSA, glucose SA medium (36); GM17, glucose M17 broth; WP, whey permeate.

[b] uninduced expression level below detection limit

[c] +, not quantified [d] determined from a *lacZ* transcriptional fusion

These repeats were proposed to have a regulatory role as a negatively acting *cis*-element. This was confirmed by deletion of the CIRCE upstream of the *L. lactis dnaJ* promoter, which resulted in temperature independent expression of a *dnaJ*::*amyS* fusion (79). Similar results were obtained by introducing point mutations in either arm of the CIRCE of *Bacillus subtilis dnaK* (87). Other workers have shown that ORF39 (homologous to ORF1 in *L. lactis* and now named *hrcA*) is a negative regulator of *dnaK* and *groE* in *B. subtilis* and that an *Escherichia coli* extract containing *B. subtilis* ORF39 protein can specifically retard a DNA fragment containing the CIRCE element in a gel shift assay. These observations were combined in a model predicting that ORF39 protein in *B. subtilis* interacts as a repressor with its operator, the CIRCE element, allowing a low level of expression at low temperature. After a temperature upshift, the repressor is inactivated and opens the way for high level expression of the heat shock operon. The transient expression after heat shock suggests that the repressor resumes interaction with the operator (31, and references therein).

Another regulator of heat shock genes in *L. lactis* may be *recA*. A culture of a *recA* mutant shifted to 37?C showed a lag of 8 hr before growth resumed at a low rate (16). Two- to threefold reduced levels of DnaK, GroEL and GrpE were observed in the *recA* strain and induction of these proteins by heat shock was delayed. In *E. coli,* the expression of most heat shock genes is controlled by the transcription factor $?^{32}$. The level of $?^{32}$ is negatively regulated by HflB, which is also an HSP. An analogue of HflB has been identified in *L. lactis* (called FtsH, 62) and its expression is induced at high temperatures (16). An HflB mutant was unable to grow at elevated temperature (38?C, 62). HflB levels were threefold higher in the *recA* strain at both 30?C and 37?C. The reduced levels of the HSPs in the *recA* mutant may be caused by the higher level of HflB in this strain. In a model based on these observations, *recA* is proposed to regulate the heat shock response via HflB that, in turn, may govern the stability of an unknown positive factor which regulates HSP expression, as is the case in *E. coli* (16, 32). However, at present there are no indications of the existence of such a positive factor, either or not a sigma factor, in *L. lactis*. Therefore, it is still unclear what other control circuits regulate HSP expression in *L. lactis* and what the exact roles of RecA and HflB are. The expression of the HSPs is also induced under other stress conditions such as low pH and UV radiation (Table 2, 28, 29, 30), but not by phage infection (85). Induction of HSPs by UV light points to the relevance of r*ecA* in HSP expression.

2.2 LOW TEMPERATURE

Whereas growth at high temperatures is deleterious to a cell, growth at low temperatures slows down biological processes. The doubling time of a lactococcal culture increased from 48 min at 30?C to 3½ hr, 57 hr and 7 days at 16?, 8?, and 4?C, respectively (64). The survival of *L. lactis* increased at low temperature. Survival of bacteria first grown at 30?C to stationary phase and subsequently incubated at 4?C for 28 days was 30%, whereas 0.03% of the culture survived when held at 30?C throughout the experiment (64). Similarly, survival of a freezing-thawing treatment was better (95%) after preincubation at 8? C for 48 hr than without such an adaptation (75%) (65). Adaptation at 16? or 4?C did not increase freezing-thawing survival significantly. Surprisingly, preincubation at 8?C for 48 hr improved survival of a 30 min-challenge at 52?C 60-fold compared to a non-adapted culture (65). Incubation at low temperature resulted in the induction of a specific set of 12 proteins in *L. lactis.* (64). The level of induction depended on the incubation time and temperature. The maximum observed overexpression of one of the proteins was tenfold after 1 hr at 8?C when compared with 30?C.

After a cold shock treatment, *E. coli* and *B. subtilis* overexpress several proteins (reviewed by Jones(39) and Graumann (25)). Research on cold shock responses was focused on a family of small (7-kDa) highly conserved cold shock proteins (CSPs), three of which were identified in *B. subtilis* and 7 in *E. coli* (25). Only 3 of the latter 7 are induced at low temperature. The function of the remaining 4 is unclear. Expression of one of the cold induced proteins, CspA from *E. coli*, was shown to be induced 200-fold at low temperature by a combination of increased transcription, increased mRNA stability and increased translation efficiency (4, 24). CspA can bind to RNA with a broad sequence specificity and increases the susceptibility to RNases (38). Therefore, CspA was suggested to prevent the formation of secondary structures in RNA molecules at low temperatures and in that way to stimulate translation efficiency. The only *csp* deletion mutant described thus far, *cspB* of *B. subtilis,* showed a defective cold shock response (25). Induction at low temperature of 15 proteins was affected in this mutant, suggesting a regulatory function for *cspB* in the cold shock response.

Recently, a study was started to identify members of the CSP family in *L. lactis*. A set of primers designed on the basis of conserved regions in the known CSPs was used to amplify an internal fragment of a lactococcal *csp* gene by PCR (J.W. Sanders, unpublished results). This cloned PCR fragment hybridized with 4 chromosomal *Hin*dIII restriction fragments (86). Three of these were cloned and

revealed the presence of 4 *csp* genes, named *cspA, cspB, cspC* and *cspD. cspB* and *cspC* were present on one fragment and were separated by 360 bp. The deduced amino acids sequences of these 4 genes show 50 to 70% identity to *E. coli cspA* and *B. subtilis cspB*. The lactococcal CSP's have from 65 to 90% identical amino acid residues. Expression of *cspA* is 40-fold induced after 4 hours at 10?C, as shown by Northern hybridization. Expression of *cspB* is 8-fold higher after 2 hr at low temperature and *cspC* expression reaches an optimum of 30-fold induction 4 hr after a shift to low temperature (10°C). *cspD* expression appeared to be temperature-independent (86). All transcripts were about 320 nucleotides (nt) in length, indicating that all genes, including *cspB* and *cspC,* are monocistronic. The start points of the *csp* mRNAs have been mapped. The nucleotide sequences of the 80-nt non-translated leaders of the messengers of the three cold inducible *csp* genes are highly conserved especially in the first 10 to 30 nucleotides. This may point to a regulatory function, as was found for the 5'-end untranslated region of the *E. coli cspA* messenger (37). From another lactic acid bacterium, *Lactobacillus plantarum*, the two *csp* genes *cspL* and *cspP* have been cloned and sequenced (56). The 66-amino-acid-proteins encoded by these genes differ by only 8 amino acids and show about 66% identity to *E. coli* CspA. Two distinct transcripts of 330 and 760-nt were detected with a *cspL*-specific probe, whereas a single 330-nt RNA was detected with a *cspP*-specific probe. A three- to fivefold increase of all three transcripts over the basal level at 37?C was observed after 1 hr at 10?C. Messenger RNA levels declined to preshock levels within one hour after return to 37?C.

2.3 OSMOTIC STRESS

Bacterial cell envelopes are permeable to water. Therefore, an increase in the osmolarity of the growth medium would result in rapid efflux of water from the cytoplasm. To retain water in the cell and, thus, to maintain turgor pressure, bacteria have systems to accumulate specific solutes that do not interfere with cell physiology (9). Such compatible solutes are either taken up from the environment or newly synthesized in the cytoplasm. One of the compatible solutes mostly used by bacteria is glycine betaine (betaine). Growth of *L. lactis* was shown to be inhibited at high salt concentrations, but growth under these conditions was stimulated considerably by the presence of betaine in the medium (58). Cells grown in CDM in the presence of 0.5 M KCl accumulated high levels of proline, and to a lesser extent aspartate. In the same medium in the presence of betaine, cells accumulated aspartate, glutamate and betaine but no proline. *L. lactis* has a high-

affinity uptake system for betaine that is constitutively expressed. In addition, a low-affinity proline uptake system was found only in CDM medium of high osmolarity and not in rich media or in the absence of KCl. Proline transport was inhibited by betaine and exchange of proline for betaine was also observed, suggesting that the proline transport system may also transport betaine. This would explain the observed absence of proline in the cytoplasm when betaine is present in the medium. Uptake by both transport systems is energy dependent but, most likely, not driven by the proton motive force (58). Information on the regulation or the genetic determinants of these systems is not available. Growth of *Lactobacillus plantarum* at high osmotic pressure is stimulated by betaine, which is the preferred osmolyte of this organism (22). Levels of alanine, glutamate, proline, and glycine increased 3-, 6-, 35-, and 48-fold, respectively, upon growth in defined medium with 0.8 M KCl compared to low-osmolarity medium, with glutamate and proline being the most abundantly accumulated amino acids. The four amino acids accumulated to lower levels when betaine was present in the high-osmolarity medium. Betaine transport was increased threefold in cells cultured in high-osmolarity medium, suggesting enhanced expression of the transport system. Uptake rates also increased when the osmolarity of the assay buffer was raised, which points towards activation of the transport protein. Efflux of betaine upon osmotic downshock depended on the post-shock osmolarity of the medium. Separate transport systems were postulated to exist for the uptake and efflux of betaine (23).

In a search for environmentally regulated genes in *L. lactis* an NaCl-inducible promoter structure was identified that was independent from the medium osmolarity but required chloride for induction (this thesis, chapter 4). The two genes transcribed from this promoter function in low pH survival. One of these, *gadC,* may code for a glutamate - ?-aminobutyrate antiporter but the involvement of these genes in osmoregulation, if any, is still unclear.

An *L. lactis ftsH* mutant is unable to grow in M17 medium in the presence of 4% NaCl and grows very slowly in 1% NaCl whereas growth of the wild-type is only reduced at the high NaCl concentration (62). Interestingly, the pattern of membrane-associated proteins in the *hflB* mutant is different from the wild-type, suggesting improper assembly of membrane proteins, some of which are essential for salt-tolerance. *hflB* expression is not induced at high osmolarity (62).

2.4 OXIDATIVE STRESS AND DNA DAMAGE

L. lactis is an obligatory fermenting bacterium. It can tolerate oxygen as its growth is not affected by aeration (76, this thesis, chapter 2). Cells are able to use oxygen

in the presence of a carbon source by a closely coupled NADH oxidase/NADH peroxidase system. This is an alternative way to regenerate NAD next to pyruvate metabolism. Pyruvate is then available for conversion to acetate, yielding extra ATP. Therefore, aerobic fermentation produces different (amounts of) end products as compared with anaerobic fermentation (76, 26). The activity of NADH oxidase/NADH peroxidase is about fivefold higher in galactose-grown aerated cells than in non-aerated cells. This enzyme activity may generate the highly toxic oxygen intermediate superoxide (O_2^-). Twofold higher levels of superoxide dismutase (which removes O_2^-) were found in aerated cultures (26; this thesis, chapter 2). The toxicity of O_2^- is illustrated by the markedly reduced growth rate of a superoxide dismutase (*sodA*) mutant during aerobic growth (this thesis, chapter 2). The remaining systems can still cope with O_2^- to a certain extent. Alternative reducing capacity may be provided by glutathione of which a relatively high level is present in *L. lactis* (18).

Both NADH oxidase and superoxide dismutase produce the toxic compound H_2O_2. *L. lactis* has no catalase and depends solely on NADH peroxidase to keep H_2O_2 levels at subinhibitory concentrations. At sublethal H_2O_2 levels, *L. lactis* develops an adaptive response against lethal concentrations of H_2O_2 (7). The *L. lactis recA* gene clearly plays a role in oxidative stress. Expression of *recA* was induced in aerated cultures and a *recA* mutant is highly sensitive to aeration, as evidenced by a lower growth rate and reduced viability during stationary phase (14). The doubling time of an aerated *recA* culture is restored to that of a non-aerated culture by the presence of catalase or the Fe^{2+} chelator ferrozine, while catalase also improves survival. This observation points to the involvement of hydroxyl radical (?OH), the most reactive oxygen species, because this is formed in the H_2O_2- and Fe^{2+}-dependent Fenton reaction ($H_2O_2 + Fe^{2+} + H^+$? ?OH + H_2O + Fe^{3+})(14). ?OH may be the cause of the observed higher rate of DNA damage in aerated cells as compared to standing cultures (16). In E. coli, O_2^- caused an increase in the intracellular pool of free iron, which promoted the rate at which H_2O_2 caused DNA damage (41).

RecA is the key protein in the SOS response to DNA damage and in homologous recombination in *E. coli*. In parallel, recombination in *L. lactis recA* was more than 10^4-fold lower than in the wild-type and the mutant was sensitive to DNA damage induced by UV light, mitomycin C or methyl methane sulphonate (16). The latter compounds also induce increases in the levels of *recA* mRNA (14), but an increase in RecA protein levels does not occur upon introduction of DNA damage (72). *recA* in *L. lactis* is cotranscribed with a gene encoding the DNA repair enzyme formamidopyrimidine DNA glycosylase (*fpg*, 14). Like its *E. coli*

counterpart, Fpg from *L. lactis* has DNA glycosylase activity and can nick DNA at abasic sites. Furthermore, it suppresses an *E. coli* mutator phenotype (15).

The lactococcal *lacX* and *lacN* genes were found to complement some defects of an *E.coli recA* strain. LacX and LacN may be a sensor and response regulator of a two component regulatory system with a function in the SOS response, but their function in *L. lactis* is unknown (34).

2.5 LOW pH

Lactic acid bacteria produce lactic acid during sugar fermentation. This implies that they are regularly confronted with acid stress. It is important to note that lactic acid is a weak organic acid that is not charged at low pH and can easily pass the cell membrane in the protonated form. At cytoplasmic pH, it dissociates and, thus, poses a stronger stress to cells at a given extracellular pH than for example hydrochloric acid (40). The intracellular pH of *L. lactis* cells in suspension was slightly reduced (from 7.0 to 6.0) when the extracellular pH was reduced with HCl (from 6.75 to 5.0) (8, 71) but decreased linearly (from 7.0 to 5.25) with the extracellular pH when that was adjusted with lactic acid (8). When measured in growing cultures, the intracellular pH decreased with the extracellular pH, by which a constant ?pH of 0.7 units was maintained (8). *L. lactis* can resist pH 4.5 (with HCl) in minimal medium, but its viability rapidly decreases by incubation at pH 4.0 (72). Cells can survive this low pH when adapted to a sublethal pH (5.5) for only 15 min, both in a defined medium adjusted with HCl (72) or in M17 medium with lactic acid (29). Acid adaptation was shown to be chloramphenicol sensitive in *L. lactis* subsp. *cremoris* MG1363 (72) but chloramphenicol-independent in *L. lactis* subsp. *lactis* IL1403 (29). Therefore, the latter strain seems to be acid resistant without the need for *de novo* protein synthesis. Incubation at pH 5.5 for 30 min triggered the synthesis of 33 proteins, among which were DnaK and GroEL (29).

A number of mechanisms have been shown to confer acid resistance. The primary mechanism for control of intracellular pH is the proton translocating F_0F_1-ATPase. Both the expression level and the activity of this protein complex are increased at low pH (45, 59). *L. lactis* mutants with reduced F_0F_1-ATPase activity were more acid-sensitive (77).

A second mechanism for pH homeostasis is the arginine deiminase pathway. This pathway enables *L. lactis* to neutralize its environment by NH_3 production (55). It consists of 3 cytoplasmic proteins: arginine deiminase, ornithine carbamoyltranferase and carbamate kinase, that catalyse the reaction: arginine + H_2O +ADP + P_i ? ornithine + CO_2 + NH_3 + ATP. These enzymes are active at low pH (2 to 3) in several *Streptococcus* species (5). Arginine and ornithine are

exchanged without the need for metabolic energy by a membrane-located antiporter. The activity of the pathway was induced 3 to 5-fold in the presence of arginine (69). Recently, genetic analysis of the ADI pathway in *Lactobacillus sake* revealed the presence of five genes (*arcABCXD*) encoding the four components of the pathway and a fifth gene with unknown function (88). Transcription of this pathway is induced by arginine and repressed by glucose (89).

L. lactis expresses a glutamate-dependent acid resistance mechanism in the presence of chloride (this thesis, chapter 4). The system is encoded by an operon consisting of two genes, *gadC* and *gadB*, which specify a putative glutamate - ?-aminobutyrate antiporter and a glutamate decarboxylase, respectively. The combined action of these two proteins may confer acid resistance by the removal of a proton from the cytoplasm and the export of ?-aminobutyrate which is more basic than the imported glutamate. Alternatively, it may also result in the formation of a proton motive force. Expression of *gadCB* was higher in unbuffered medium, resulting in a lower pH at the end of growth, than in buffered medium and may also depend on glutamate (this thesis, chapter 4). Similar mechanisms, based on the combination of amino acid antiport and amino acid decarboxylation have been described for lactobacilli. An aspartate-alanine antiporter that could stimulate ATP formation was described in *Lactobacillus* subsp. M3 (1). *Lactobacillus buchneri* is able to generate a proton motive force by histidine decarboxylation and electrogenic histidine-histamine antiport (57). Interestingly, a histidine decarboxylase mutant of *Lactobacillus* 30a was unable to alkalinize its environment in the presence of histidine (73), suggesting that these systems may support acid survival by restoring the pH to levels which permit growth.

In a transposon random insertion study, clones were selected that express the transposon-encoded *lacZ* reporter at a higher level in response to low pH (35). One of these, PA170, showed 50-fold higher *lacZ* expression in a chemostat maintained at pH 5.2 than at pH 7.0. Expression was only observed in the stationary phase and was also induced 7-fold at 15?C compared to 30?C. Genetic analysis of this promoter revealed that a 50-bp region is sufficient for transcription initiation in response to all three inducing conditions (54). Removal of a part of the DNA specifying the untranslated 5'-end of the transcript resulted in a 100-fold higher level of pH-regulated expression. This same fragment could also reduce the expression of an unrelated gene when it was introduced at its 5'-end. The function of the gene transcribed from the P170 promoter is not known (54).

To study regulation mechanisms that control acid resistance, acid resistant IS*S1* insertion mutants were selected (72). One of the genes identified in this way shows homology with *ahrC*, a regulator of arginine synthesis and catabolism in *B.*

subtilis, and may be involved in control of the arginine deiminase pathway. A number of the other acid resistant mutants contained insertions in genes that have roles in nucleotide metabolism and may act in the biosynthesis of (p)ppGpp, the stringent response regulator. This result points to the possible involvement of the stringent reponse in pH regulation in *L. lactis* (72).

2.6 STARVATION

Exhaustion of an essential nutrient limits growth of a culture which then enters the stationary growth phase. It is generally accepted that stationary phase is the most common state of bacterial cells in nature. Glycolytic and arginine deiminase pathway activities declined rapidly upon entry of *L. lactis* in the stationary phase (48). These activities could be restored by the addition of sugar. Resumption of glycolytic activity was slower after a longer period in stationary phase and was chloramphenicol sensitive. In contrast, the arginine deiminase pathway turned to the same rate of activity independent from the duration of starvation and required no protein synthesis. Total cell protein is degraded during exponential growth and in the first 30-90 min of stationary phase, but proteins were stably maintained afterwards (48). Cells react to starvation by increasing the level of 14 proteins, despite the lack of metabolic energy. Major changes were also observed in the protein composition of the cytoplasmic membrane, but amino acid transport capacity was hardly affected in the stationary phase (48). Although *L. lactis* is unable to react to starvation by, for instance, the development of competence or the formation of spores, it does develop multiple stress resistance. In this way, cells are prepared to a sudden stress, to which they may be unable to quickly adapt in the stationary phase. The first sign of such an adaptation was the observation that lactose starved cells survived longer when cultured at a lower imposed growth rate before starvation (70). *L. lactis* appeared to be highly resistant to incubation at 52?C or pH 4.0, or to the presence of 20% ethanol, 3.5 M NaCl or 15 mM H_2O_2, already at the onset of stationary phase (27). Analysis of acid resistance of cells during growth revealed that it is acquired during the late exponential phase. The latter is not surprising as growth of *L. lactis* always results in acid formation.

Genetic studies on starvation response have just been started. Examples of genes expressed in the stationary phase are the nisin operon (10) and the gene controlled by promoter P170 (35).

2.7 CROSS PROTECTION AND GLOBAL REGULATION

Pretreatment with a mild stress makes cells resistant to a lethal challenge with the same stress condition, as has been discussed above for a number of conditions. Moreover, preadaptation to one stress condition can render cells resistant to other stress-imposing conditions. This is of relevance for cells in environments where they can be exposed to combinations of stress conditions. The best example of such a multiple stress resistance is that obtained in the stationary phase. Also, exposure to UV-light (100 J/m^2) conferred cross protection against a treatment with heat, acid or ethanol and, to a lesser extent, against H_2O_2 (28). Another aspect of the same phenomenon was seen by total protein analysis in 2D-gels. UV challenge induced 14 proteins of which four were also induced by other stress conditions. Similarly, nine of the 33 proteins that were induced at low pH were also induced by heat shock (29). This is suggestive of an overlap between the control circuits regulating responses to these stresses. Other evidence for the existence of such interference is the involvement of *recA* in both oxidative stress and heat shock and the acid inducibility of *ftsH* (78), which is also implicated in heat shock response. Interestingly, in a selection procedure for thermoresistant mutants, a number of the disrupted genes appeared to be the same as those found in selection for acid resistant mutants (72). This implies that the heat shock response may also in some way be controlled by the stringent response. However, there is no evidence for direct control of heat shock genes or regulators and acid resistance genes by the stringent response. Another open question as to the control of stress responses in *L. lactis* is the involvement of sigma factors.

Despite several attempts to identify alternative sigma factors, thus far only the vegetative sigma factor has been described (19). Control mechanisms in *L. lactis* are likely to resemble those in the Gram-positive bacterium *B. subtilis* more than those of the Gram-negative *E. coli*. Indeed, remarkable similarities in the control of heat shock genes, including the CIRCE element and *hrcA,* have been found between *L. lactis* and *B. subtilis* (see above). Another group of *B. subtilis* genes involved in general stress resistance is controlled by $?^B$ (31). It would be interesting to elucidate whether such general stress proteins and their regulator $?^B$ are present in *L. lactis*. The striking properties of *recA* in relation to heat shock response and oxidative stress suggest that control of stress responses in *L. lactis* is markedly different from other bacteria, as has been noted for the control of other cellular functions (46).

3 EXPRESSION SYSTEMS FOR *L. LACTIS*

A number of constitutive gene expression systems are available for *L. lactis*. However, both industrial and laboratory practice require inducible expression systems suitable for use in *L. lactis.* These systems should, preferably, consist entirely of *L. lactis* sequences. The expression of a number of lactococcal operons is regulated (reviewed in 46). Only few of the regulatory elements involved have been used for the controlled expression of other genes, as wil be discussed here.

3.1 INDUCIBLE GENE EXPRESSION SYSTEMS NOT BASED ON STRESS-INDUCING CONDITIONS

The first inducible promoter characterized in *L. lactis* is that of the *lac* operon. This promoter (P_{lac}) regulates the expression of the phosphoenolpyruvate transferase system and the enzymes of the tagatose-6-phosphate pathway. A DNA fragment of 500 bp was required for lactose inducible expression from P_{lac} (80). Expression in lactose grown cells was 5-fold higher than in glucose grown cells. Low level expression with glucose was only obtained in the presence of the repressor LacR. P_{lac} was exploited for the controlled expression of a *Listeria monocytogenes* bacteriophage lysin (66). The lysin gene was inserted in *lacG* of the chromosomally located *lac* operon and growth on lactose of this strain resulted in fourfold higher levels of anti-listerial activity as compared with glucose grown cells. The applicability of P_{lac} was also tested in a plasmid-based system. A food-grade expression vector contained *lacF* as a selection marker and was stably maintained in a *L. lactis lacF* strain (68). High levels of ?-glucuronidase were present in glucose-grown cells carrying this vector and these levels were induced twofold upon growth in lactose-containing medium. A major improvement of this inducible system was the combination of P_{lac} with the fast-acting *E. coli* bacteriophage T7 RNA polymerase (T7RNAP) gene (82). Upon lactose induction, T7RNAP is expressed and specifically transcribes genes from the T7RNAP-specific promoter. Soluble recombinant protein, tetanus toxin fragment C (TTFC), represented up to 22% of total protein in cells carrying the lactose inducible P_{lac}::T7RNAP gene fusion and a fusion of the T7 promoter with the gene encoding TTFC. The lactose-inducible T7RNAP was also used for the secretion of up to 2.9 mg/l culture fluid of TTFC, by fusing the TTFC gene to the 5'-parts of *usp45* or *prtP* encoding the secretion signal sequences of Usp45 or PrtP (83). This expression system proves that *L. lactis* is suitable for the production of high levels of a single protein but it has the disadvantage of containing heterologous genetic elements.

Recently, it was uncovered that the antimicrobiol peptide nisin is a potent inducer of expression of its own structural gene *nisA* (47). Nisin is secreted by a number of *L. lactis* strains and serves as an induction signal that is transduced by a two component regulatory system encoded by *nisK* and *nisR*. The expression level of a translational *nisA*::*gusA* fusion was proportional to the amount of extracellular nisin. In the absence of nisin no expression was detectable and an at least 1000-fold induction of ?-glucuronidase was obtained 90 to 120 min after addition of a subinhibitory amount of nisin (1ng/ml) (10). A translational fusion of *nisA* with *pepN* was used for high-level production of aminopeptidase N. PepN represented approximately 47% of total cytoplasmic protein upon induction of the fusion with 0.5 ng/ml nisin. These results show that it is possible to obtain high levels of gene expression in *L. lactis* by using exclusively lactococcal DNA.

The promoter of the *purDEK* operon, which is involved in the *de novo* biosynthesis of purine nucleotides, is repressed 35-fold by exogenous purines. Cells containing a food-grade vector with a fusion of this promoter and the lysin gene of bacteriophage ? vML3 were more prone to autolysis and produced higher levels of lysin in the absence of purines (42).

An alternative method for the production of proteins was based on DNA from a lactococcal bacteriophage. A middle promoter of the lytic bacteriophage ? 31 was isolated and combined with the ? 31 origin of replication on an expression vector (63). After infection of lactococcal cells carrying this vector with ? 31, the copy number of the plasmid was amplified and expression of the gene(s) under control of the phage promoter was induced. More than 2000 units of ?-galactosidase were produced in a phage lysate of *L. lactis* when the phage promoter was fused to *Streptococcus thermophilus lacZ,* whereas expression was negligible without infection. This high level of expression of ?-galactosidase is mainly attributable to plasmid amplification, as a vector lacking the phage replication functions expressed only 85 units of ?-galactosidase.

3.2 STRESS-INDUCIBLE GENE EXPRESSION SYSTEMS

Regulation signals of lactococcal heat shock genes have been used for heterologous gene expression. However, the levels of induction from these promoters upon heat shock were rather low (20, 44). A fusion of the *dnaJ* promoter with *usp45::amyS* showed a fourfold higher level of secreted *Bacillus stearothermophilus* ?-amylase activity 30 min after a shift from 30?C to 42?C (79).

A more suitable promoter is P_{gad}, the promoter of the lactococcal *gadCB* operon (this thesis, chapter 4). *gadCB* expression can be induced more than 1000-

fold by growth in the presence of 0.5 M NaCl. In addition, expression is induced at low pH. A positive regulator encoded by *gadR* is indispensable for the chloride dependent expression of *gadCB*. The P_{gad} promoter was amplified together with *gadR* and this expression cassette was inserted upstream of *lytPR* of the lactococcal bacteriophage r1t and *acmA* of *L. lactis.* Induction of *L. lactis* carrying these fusions with NaCl resulted in overexpression of the cell wall degrading enzymes and in cell lysis, evidenced by the release of cytoplasmic enzyme activity (this thesis, chapter 5). The basal expression level of the fusions was very low and did not harm the cells.

The genetic switch element of the temperate lactococcal bacteriophage r1t consists of two divergently transcribed genes, *rro*, encoding the phage repressor, and *tec*. Expression of the genes of the lytic growth phase downstream of promoter P_2 is repressed by Rro. When *E. coli lacZ* was placed on a plasmid downstream of P_2, ?-galactosidase expression was induced about 70-fold after addition of 1 ?g/ml mitomycin C (60). Rro was shown to bind specifically to an operator sequence present twice in the promoter region and once in *tec* and can in this way shut off expression of the P_2-driven genes. Recently, a temperature sensitive repressor mutant (Rro12) was constructed by site directed mutagenesis on the basis of comparative molecular modelling. Promoter P_2-driven expression of *lacZ* is efficiently repressed by Rro12 at 24?C and is 600-fold higher at 40?C (61).

4 OUTLINE OF THIS THESIS

The objective of this work was to identify, isolate and characterize environmentally controlled inducible gene expression signals of *L. lactis.* A second aim was to apply this sequence(s) for the inducible lysis of *L. lactis*. Stress-induced gene expression can be analysed by studying overexpression of proteins during stress, or by looking for increased transcription levels of certain genes. In the first approach, the protein composition of stressed cells is compared with that of non-stressed cells in SDS-PAA gels. The lactococcal superoxide dismutase gene (*sodA*) was cloned by reverse genetics using the N-terminal amino acid sequence of such a differentially expressed protein (chapter 2). *sodA* expression was induced twofold upon aeration. The growth rate of a *sodA* mutant was unaffected in standing cultures but was reduced in aerated cultures. This observation conforms to the role of SodA in prevention of oxidative damage to cells by removing superoxide radicals (O_2^-).

Transcription of (un)known genes can be monitored by using a reporter gene. In chapter 3 a method was developed for the random insertion of a single copy of the reporter gene *lacZ* of *E. coli* in the genome of *L. lactis* by Campbell integration, with the aim to select for stress-inducible genes. Using this system, a chloride inducible transcription signal was identified. The promoter involved (P_{gad}) is responsible for the expression of two genes, *gadC* and *gadB*, that together encode a glutamate dependent acid resistance mechanism (chapter 4). The suitability of this chloride dependent promoter for the expression of toxic gene products is shown in chapter 5 by the NaCl induced expression of cell wall degrading enzymes, which ultimately results in lysis of *L. lactis*.

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